

Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) EP 0 759 555 A2

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
26.02.1997 Bulletin 1997/09

(51) Int Cl.⁶: G01N 33/52

(21) Application number: 96305720.3

(22) Date of filing: 02.08.1996

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU NL
PT SE

(30) Priority: 03.08.1995 US 528511

(71) Applicant: LIFESCAN, INC.
Milpitas California 95035 (US)

(72) Inventors:
• Douglas, Joel
Santa Clara, CA 95051 (US)
• Tomasco, Michael F.
Mountain View, CA 94041 (US)

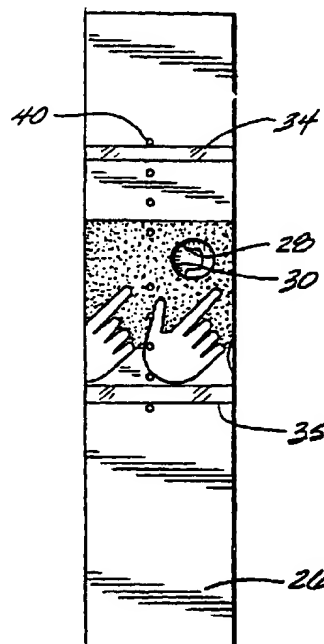
• Kiser, Ernest
Los Altos, CA 94024 (US)
• Dato, Remedios
Pleasanton, CA 94588 (US)
• Rice, Edward G.
Palo Alto, CA 94303 (US)
• Tuohy, Deborah P.
San Jose, CA 95120 (US)
• Maxson, Mark
San Jose, CA 95148 (US)

(74) Representative: Mercer, Christopher Paul et al
Carpmaels & Ransford
43, Bloomsbury Square
London WC1A 2RA (GB)

(54) Direct-reading reagent test strip

(57) A multilayer reagent test strip measures the concentration of analyte in a liquid sample that is applied to it. The sample is guided to a number of assay areas arrayed along the strip, where the analyte can react with a reagent to cause a color change. Each assay area also includes an inhibitor for the color-change reaction. The inhibitor concentration increases in successive assay areas; thus, the number of areas that change color is a measure of the analyte concentration. The test strip is particularly adapted for measuring glucose in a whole blood sample. In a preferred embodiment, the sample is guided to the assay areas along a path formed by crushing selected areas of a membrane, and the assay areas are uncrushed areas of the membrane.

FIG. 5



EP 0 759 555 A2

DescriptionBackground of the Invention5 1. Field of the Invention

This invention relates to a dry test strip for measuring the concentration of an analyte in a biological fluid; more particularly, a test strip that measures the concentration directly, without the need for a meter.

10 2. Description of the Related Art

Many visual test devices have been developed for measuring the concentration of certain analytes in biological fluids. These devices have, for example, measured glucose, cholesterol, proteins, ketones, phenylalanine, or enzymes in blood, urine, or saliva.

15 Dry phase reagent strips incorporating enzyme-based compositions are used extensively in clinical laboratories, physician's offices, hospitals, and homes to test samples of biological fluids for glucose concentration. In fact, reagent strips have become an everyday necessity for many of the nation's several million diabetics. Since diabetes can cause dangerous anomalies in blood chemistry, it can contribute to vision loss, kidney failure, and other serious medical consequences. To minimize the risk of these consequences, most diabetics must test themselves periodically, then
20 adjust their glucose concentration accordingly, for instance, through diet control and/or with insulin injections. Some patients must test their blood glucose concentration as often as four times daily or more.

It is especially important for diabetics who must control their diet in order to regulate sugar intake and/or administer insulin injections, and who must be guided in this regard by frequent tests of blood glucose concentration, to have rapid, inexpensive, and accurate reagent strips for glucose determination.

25 Reagent strips are known that contain an indicator which turns a different shade of color, depending on the concentration of glucose in a biological fluid that has been applied to the strip. Although some of these strips use reduction chemistries, more commonly they involve an oxidizable dye or dye couple. Some of the strips include an enzyme, such as glucose oxidase, which is capable of oxidizing glucose to gluconic acid and hydrogen peroxide. They also contain an oxidizable dye and a substance having peroxidative activity, which is capable of selectively catalyzing oxidation of
30 the oxidizable dye in the presence of hydrogen peroxide. (See, for example, U.S. Pat. No. 5,306,623, issued April 26, 1994, to Kiser et al.)

U.S. Pat. No. 3,964,871, issued June 22, 1976, to Hochstrasser, discloses a disposable indicator strip for directly measuring substances, such as glucose, in biological fluids. The indicator registers the concentration of the substance by including both an indicator reagent, which is oxidized and changes color when it reacts with the substance, and an
35 "antagonist" that in some way prevents the accumulation of oxidized indicator until it has been completely consumed.

Palmer et al. disclose a "digital" quantitative assay system for glucose and other analytes in European Patent Application Publication No. 0 317 070, published May 24, 1989 (see also U.S. Pat. No. 5,036,000, issued July 30, 1991). That system measures the concentration of an organic compound in a biological fluid by first oxidizing the compound with a substrate-specific oxidase enzyme to produce hydrogen peroxide. The system includes a chromogen that is a
40 reductant of hydrogen peroxide and an air-stable hydrogen peroxide reductant that has a larger reduction potential. The larger reduction potential delays any detectable color change by the chromogen until the air-stable first hydrogen peroxide reductant has been consumed. Thus no color change results if the hydrogen peroxide to be measured is less than a pre-determined level corresponding to the concentration of the air-stable peroxide reductant. As a result, the system measures the concentration quantitatively, independent of color change intensity.

45 Englemann, U.S. Pat. No. 4,738,823, issued April 19, 1988, discloses a test strip for analyte determination that has a support member, which has an absorbent material positioned to remove excess sample applied to the strip. The strip may also include a cover, which includes an opening through which sample may be introduced.

Burkhardt et al., U.S. Pat. No. 4,810,470, issued March 7, 1989, disclose a device for measuring analyte concentrations in liquid samples. The device includes one or more bibulous matrices covered by a liquid impervious coating
50 or film. The sample is deposited on a portion of a bibulous matrix and is metered into the matrix chromatographically. By wicking action, the sample travels to an assay region that contains a test reagent for the analyte.

Daffern et al., U.S. Pat. No. 4,994,238, issued February 19, 1991, disclose a chemical analysis test device that comprises an absorbent layer, a waterproof barrier layer, and a reagent layer that has a determinate volume. The sample is applied to the reagent layer through aligned holes in the overlying absorbent and barrier layers.

55 Whether the test is conducted in the home, physician's office, clinic or a hospital, accuracy and reproducibility of a glucose determination are extremely important. In the case of a color-indicating reagent strip, it is desirable that the color change be pronounced and insensitive to variations in components of the biological fluid other than glucose. In the case of a visually-read reagent strip, it is especially important that diabetics, who may have impaired vision, have

a strip that exhibits a significant color change dependent upon glucose concentration, although color change as exhibited by a change in absorbance at a given wavelength is also important for the accuracy of meter-read strips.

Since the color change involves a series of chemical reactions, it doesn't happen instantaneously. Thus, the user must wait a period of time - typically a minute or less - for the reactions to take place. When a meter reads the strip, timer circuitry can give a signal that indicates the reactions are completed. However, when a strip is read visually, without a meter, the user may underestimate the time needed, read the strip prematurely, and get an incorrect result. Alternatively, the user may feel the need to wait an excessive time before reading the strip, to be sure the reaction is complete, causing unnecessary delay and user dissatisfaction. There is thus a need for a "chemical" timer; i.e., an element on the strip that will change color regardless of the concentration of glucose (or other analyte of interest) in the sample, but will do so only after sufficient time has passed to complete the color-forming reactions with the sample.

Summary of the Invention

In accordance with the present invention, an elongated multilayer reagent test strip for measuring the concentration of analyte in a sample of biological fluid that is applied to the strip comprises

- a) a bottom layer with a through hole for accepting the sample;
- b) a membrane layer, having a sample side facing the bottom layer and a testing side opposite to it, and containing a reagent that can react with the analyte to produce a color change, the reagent comprising

- i) a first component that interacts with the analyte to form hydrogen peroxide;
- ii) a second component that interacts with the hydrogen peroxide to undergo a color change; and
- iii) a third component that inhibits the change in color of the second component;

- c) an intermediate layer between the bottom and membrane layers; and
- d) metering means for distributing sample along the strip, the metering means comprising

- i) a non-bibulous region in the membrane layer; and
- ii) a fluid transport channel formed in the intermediate layer for guiding sample over the surface of the non-bibulous region to a plurality of discrete bibulous assay areas arrayed along the length of the membrane layer;

the inhibitor concentration increasing in a predetermined way with distance from a first end of the strip, so that a correspondingly increasing analyte concentration must be contained in a sample if it is to effect a color change, whereby one or more assay areas may change color when a sample is applied to the strip, and the color-changing area most distant from the first end indicates the analyte concentration in the sample.

In operation, a method for measuring the concentration of analyte in a sample of biological fluid, comprises the steps of:

- (a) applying the sample to a reagent test strip that comprises:

- (i) a plurality of bibulous assay areas that each change color when contacted with fluid containing at least a predetermined amount of analyte, greater than the amount of analyte that causes a change in color of the assay areas that are closer to a first end of the strip and
- (ii) metering means for distributing the sample along a predetermined non-bibulous path to each of the assay areas and

- (b) observing the assay area that changes color and that is most distant from the first end of the strip.

The strip is of the type that provides a visible indication of the concentration of an analyte that is contained in a biological fluid applied to a "sample side" of the strip. The visible indication appears on the opposite (or "testing") side of the strip.

The chemical composition of the test strip depends, of course, on the analyte/biological fluid to be measured. Test strips can be designed to detect analytes such as glucose or other sugars, alcohol, cholesterol, proteins, ketones, uric acid, phenylalanine, or enzymes in biological fluids such as blood, urine, and saliva, as well as water. For the sake of convenience and brevity, the reagent test strips disclosed in the most detail in this specification detect glucose in blood. A person of ordinary skill in the art could readily adapt the information in this disclosure for detecting other analyte/biological fluid combinations.

A test strip of the present invention provides a relatively simple and rapid determination of glucose concentration

in an unmeasured sample of blood. The strip comprises a bottom layer with a hole through which a sample may be introduced to the sample side of a porous matrix, whose opposite side is the testing side. The matrix is generally a membrane and the two terms are used interchangeably in the present specification and the appended claims. A testing reagent is applied to the matrix and, to a greater or lesser extent, is impregnated within the pores of the matrix. For simplicity, we sometimes refer to the reagent on the matrix as a "coating", in this specification and in the appended claims, recognizing that the reagent coating penetrates the matrix.

An intermediate layer lies between the bottom layer and the matrix. Cutouts in the intermediate layer align with non-bibulous areas of the membrane to guide the sample to a series of bibulous assay areas that are arrayed along the strip. (As used in this specification and the appended claims, "bibulous" is understood to mean absorbent.) A series of notches in the intermediate layer surround the space around and above the assay areas to constrain the flow of sample to these areas.

A fixed volume of sample - typically whole blood containing red cells and glucose - is thus directed to the sample side of the membrane at each of a series of assay areas. Preferably, the bottom layer has vent holes in alignment with the areas to facilitate filling the volumes uniformly. The porosity of the matrix permits fluid to pass from the sample side toward the testing side, for example by capillary action. Thus, the testing reagent can react with glucose in the blood to cause a color change on or near the testing side. Since the strongly-colored red cells can make it harder to detect the color change, the matrix is preferably anisotropic, with pore sizes graduated from large pores on the sample side to smaller pores on the testing side, in order to trap red cells away from the testing side. A variety of materials may be used for the various components of the test strip and timer of this invention. Some of these materials are disclosed in U.S. Pats. 5,306,623 and 5,418,142, issued April 26, 1994 and May 23, 1995, respectively, to Kiser et al., and incorporated herein by reference.

The testing reagent comprises a component for converting glucose to hydrogen peroxide, such as glucose oxidase; one or more components for detecting the hydrogen peroxide produced from the glucose present in the sample; and an inhibitor. The components for detecting hydrogen peroxide may be a peroxidase, preferably horseradish peroxidase, together with an "indicator" that changes color in the course of the reaction. The indicator may be an oxidizable dye or a dye couple. The peroxidase catalyzes the oxidation of the indicator in the presence of hydrogen peroxide. The final element of the reagent is an inhibitor that retards the color-changing oxidation of the indicator.

The strip is segmented along its length in such a way that adjacent membrane segments have different inhibitor concentrations. Each segment has a bibulous assay area that only changes color if and when enough glucose is present to first cause all the inhibitor to be consumed and to then oxidize the indicator and thereby cause the characteristic color change. Thus, a color change in a particular area evidences a threshold glucose concentration in the original blood sample. Along the strip, in a particular direction, each successive segment has a stepwise greater inhibitor concentration, which corresponds to a stepwise increase in threshold glucose concentration. The indicator concentration is the same for all segments. In principle, other varying inhibitor/indicator balances are also possible.

If the segments have inhibitor concentrations in the appropriate range for a particular test sample, adjacent assay areas react with the analyte such that one area is colored and an adjacent one is not. That result indicates that the glucose concentration in the sample is at least equal to the threshold concentration required to change the color of the one area, but not as great as that required to change the color of the adjacent area.

For blood glucose monitoring, an optional timer segment coating comprises the elements of the indicator strip - a porous matrix having a testing reagent coated on it - and, in addition, glucose. In the dry state, the reagent chemistry is not activated by the glucose, but when a sample is applied to the strip, the timer coating is hydrated and the glucose in the coating, after a predetermined time, causes the indicator to change color. Preferably glucose is present in the timer in an amount well in excess of that required to overcome the inhibitor. In that case, the time required is longer or shorter depending on whether more or less inhibitor is present. Color changes in the strip and in the timer can be observed either directly by the eye or with an optical instrument that detects changes in reflectance.

Brief Description of the Drawings

FIG. 1 is a perspective view of the matrix of a direct-reading reagent test strip of the present invention.

FIG. 2 is a cutaway bottom plan view of the sample side of a direct-reading reagent test strip of the present invention.

FIG. 3 is an enlarged fragmentary perspective view of the interior of the test strip of FIG. 2, partially cutaway.

FIG. 4 is a cross-section of the strip of FIG. 2 taken along line 4-4.

FIG. 5 is a bottom plan view of a test strip of this invention.

FIG. 6 is a top plan view, showing the testing side of the test strip of FIG. 5.

FIG. 7 is the strip of FIG. 6 after a sample has been applied to it.

Detailed Description of the Invention

The present invention is a direct-reading reagent test strip for measuring concentration of an analyte in a biological fluid. The key element of such a test strip is a porous matrix that incorporates a testing reagent that undergoes a color change in response to the analyte in a biological fluid sample that is applied to the strip.

The matrix may be of a uniform composition or may be a coated substrate and may be either isotropic or anisotropic. It has a sample side, to which the sample is applied, and a testing side, where the color change is observed. Preferably, the matrix is an anisotropic membrane; more preferably, an anisotropic membrane having a broad range of pore sizes. For example, a gradient of pore sizes from about 0.1 micrometers to about 150 micrometers may extend through the membrane. On the large-pore side, pore size is preferably in the range from about 30 micrometers to about 40 micrometers. On the side of the membrane where the pores are smallest, the void volume is relatively small, and the material of the membrane is generally quite dense, within a layer that can typically constitute up to 20% of the membrane's thickness. Within this layer, pore size is preferably in the range from about 0.1 to about 0.8 micrometers, with a nominal pore size preferably about 0.3 micrometers. When the biological fluid is applied to the sample side, the sample encounters increasingly smaller pores as it penetrates the membrane. Eventually, solids such as red blood cells reach a position in the membrane where they can penetrate no further. The balance of the sample, still containing the dissolved glucose, penetrates through to the testing side. The anisotropic nature of the membrane and/or use of a separating component (discussed below) permits relatively rapid flow rates through the membrane, even while filtration of the solids is taking place.

As the sample passes through the matrix, reaction with the reagent causes a light-absorbing dye to be formed or decomposed in the void volume near the testing side, thereby substantially affecting reflectance from the matrix.

Polysulfones and polyamides (nylons) are examples of suitable matrix materials. Other polymers having comparable properties may also be used. The polymers may be modified to introduce other functional groups which provide for charged structures, so that the surfaces of the matrix may be neutral, positive, or negative.

A preferred method of preparing the porous material that forms the matrix is to cast the polymer without a supporting core. Such a matrix is, for example, the anisotropic polysulfone membrane available from Memtec, Inc., Timonium, MD. A matrix of less than about 200 micrometers thickness is usually employed, with about 115 to 155 micrometers being preferred. A thickness of about 130 to 140 micrometers is most preferred, particularly when the matrix is nylon or anisotropic polysulfone.

The membrane may be treated with testing reagent by dipping it into an admixture of the components, thereby saturating the membrane. Preferably, at least some of the components are applied to the membrane sequentially. Excess reagent may be removed by mechanical means such as, for example, an air knife, doctor blade, or glass rod. The membrane is then dried. Reagent tends to concentrate near the small-pore (testing) side of the membrane.

The testing reagent comprises (i) a component for converting glucose to hydrogen peroxide, (ii) a component for detecting hydrogen peroxide, and (iii) a component for inhibiting the component that detects the hydrogen peroxide. The reagent may optionally further comprise a separating component which causes solids, such as red blood cells, to become entrapped in the matrix, effectively removing the solids from the biological fluid. Additional components may also be included as described hereinbelow and in the Examples.

Preferred components for converting glucose to hydrogen peroxide include glucose oxidase, an enzyme that is usually obtained from *Aspergillus niger* or *Penicillium*. Glucose oxidase reacts with glucose and oxygen to produce gluconolactone and hydrogen peroxide. Optimum glucose oxidase concentration depends on the composition of the indicator system. For example, if the indicator system is MBTHSB-ANS (which is described below), then glucose oxidase in the range from about 500-10,000 U./mL is suitable, more preferably from about 700-2000 U./mL, and most preferably about 1000 U./mL. Generally, higher concentrations of glucose oxidase cause the reaction to proceed more rapidly and lower concentrations, less rapidly.

The hydrogen peroxide so produced reacts with the component for detecting hydrogen peroxide, which comprises a peroxidase that selectively catalyzes a reaction between the hydrogen peroxide and an indicator. The peroxidase uses hydrogen peroxide as an oxidant which is capable of removing hydrogen atoms from various substrates. A suitable peroxidase may contain ferriprotoporphyrin, a red hemin obtained from plants. Peroxidases obtained from animals, for example from the thyroid glands of animals, are also suitable. Horseradish peroxidase (HRPO) is especially preferred as a constituent of the component for detecting hydrogen peroxide.

The hydrogen peroxide, preferably catalyzed by a peroxidase, reacts either directly or indirectly to form or decompose an indicator dye that absorbs light in a predetermined wavelength range. Preferably, the indicator dye absorbs strongly at a wavelength different from that at which the testing reagent absorbs strongly. The oxidized form of the indicator may be the colored, faintly-colored, or colorless final product that evidences a change in color of the testing side of the matrix. That is to say, the testing reagent can indicate the presence of analyte in a sample by a colored area being bleached or, alternatively, by a colorless area developing color.

Indicators that are useful in the present invention include (a) 3-methyl-2-benzothiazolinone hydrazone hydrochloride

ride (MBTH) combined with 3-dimethylaminobenzoic acid (DMAB); (b) MBTH combined with 3,5-dichloro-2-hydroxybenzene-sulfonic acid (DCHBS); (c) 4-aminoantipyrine (4-AAP) and 5-oxo-1-(p-sulphophenyl)-2-pyrazoline-3-carboxylic acid (OPSP); (d) 4-AAP and N-(m-tolyl)-diethanolamine (NDA); (e) 2,2'-azino-di (3-ethylbenzthiazoline) sulfonic acid (ABTS); (f) 4AAP and 4-methoxynaphthol; (g) pyrogallol red (PGR); (h) bromopyrogallol red (BPR); (i) Acid Green 25 (AG); or (j) [3-methyl-2-benzothiazolinone hydrazone] N-sulfonyl benzenesulfonate monosodium (MBTHSB), combined with 8-anilino-1-naphthalene sulfonic acid ammonium (ANS). MBTHSB-ANS is preferred. Additional information regarding MBTHSB-ANS appears in copending U.S. Pat. Appl. Serial No. 302,575, filed on September 8, 1994 and incorporated herein by reference.

The inhibiting component retards the reaction between the hydrogen peroxide and the indicator, for example by reducing the hydrogen peroxide or by reducing the oxidized indicator. In principle there are several different modes of operation for an inhibitor. First, the inhibitor could compete with the indicator and thereby slow the rate at which the color change takes place in the indicator. Second, the inhibitor could be non-competitive, so that substantially all the inhibitor is consumed before any substantial color change takes place in the indicator. Other modes of inhibitor operation are also possible. Preferably, inhibitors of the present invention are non-competitive.

Among the range of suitable inhibitors are 2, 3, 4-trihydroxybenzoic acid; propyl gallate; 3, 4 dihydroxy cinnamic acid; 3,4 dihydroxy benzaldehyde; gallic acid; 5, 6-diaminouracil; ascorbic acid; and isoascorbic acid. Ascorbic acid is preferred; however, ascorbic acid oxidizes in solution and must be stabilized in order to permit the reagent to be coated. Preferred stabilizers are primary alcohols, such as ethyl, methyl, or propyl alcohol. Ethyl alcohol is preferred, particularly concentrated solutions; i.e., solutions of 50% or more ethanol.

Although the anisotropic membrane that is the preferred matrix filters out red blood cells and holds them away from the testing side, optionally the testing reagent may also contain a separating component. The separating component should be capable of producing a relatively clear colorless fluid from fluid containing red blood cells, e.g., whole blood, by sequestering red blood cells in the matrix. Separating components for use in the instant invention include but are not limited to polyethylene glycol, poly (methylvinyl ether/maleic) anhydride, polypropylene glycol, polystyrene sulfonic acid, polyacrylic acid, polyvinyl alcohol, and polyvinyl sulfonic acid at a pH of between about 4.0-8.0. Such separating components are present in the matrix in amounts that will vary depending upon their charge and molecular weight, the other components imbedded in the matrix, the matrix pH and pore size, and the residual moisture of the matrix after drying. Such parameters are readily determinable by one skilled in the art. For example, when polypropylene glycol is employed as the separating component (e.g., PPG-410 from BASF, Wyandotte, MI), it is preferably present at about 2-30% weight to volume (w/v), and more preferably 8-10% w/v. Other separating components can also be employed in a concentration of about 2-30% w/v. The polymeric separating components may be impregnated or imbedded in the matrix or cast in the membrane during manufacture.

Some water soluble salts can also effect blood separation. Among salts suitable for separating blood components are citrates, formates, and sulfates, as well as certain acids, such as amino acids, citric acid, phytic acid, and malic acid. (See, e.g., U.S. Pat. 3,552,928, issued January 5, 1971, to M.C. Fetter.) An advantage of including the separating component is that with solids such as red blood cells substantially removed from the biological fluid, there is less background color at the test site to obscure a change in coloration produced by the testing reagent.

Other components may be imbedded into the matrix to enhance the coloration and readability of the reagent strips and to preserve the uniformity and integrity of the matrix. For example, the testing reagent may include salts and/or buffers to aid in the separation of the dye in the matrix. Such buffers may contain for example, citrate, present in solution at from about 0.01M to about 1.0 M, and preferably at about 0.1M. Other buffers may also be employed.

Compounds that make the matrix hydrophilic or compounds that can act as stabilizers, such as hydrolyzed proteins, may also be employed. Such compounds include but are not limited to for example bovine serum albumin, polypeptides and the low molecular weight protein available as Crotein SPA (CRODA, Inc. New York, N. Y.). Such compounds are used at concentrations of for example about 1 mg/mL to about 100 mg/mL. In the case of Crotein, about 30 mg/mL is preferred.

Other stabilizers and preservatives may also be included in the coating for the matrix. For example ethylene diamine tetraacetic acid (EDTA), diethylene triamine pentaacetic acid (DTPA) and related compounds may be employed, for example, at concentrations of about 0.01 mg/mL to about 10 mg/mL. Among the purposes of the preservatives is to help to stabilize the inhibitor.

Some of the indicators (e.g., BPR) have an undesirable tendency to migrate in the matrix. When such an indicator is used, an ion pairing agent is included to prevent such migration. For example, the polyethylene glycol derivatives commercially available as Polyquart (H) (Henkel, Inc., Ambler, PA) are particularly useful for their ability to facilitate ion pairing between the indicator and other matrix substituents.

When the presence of an analyte is indicated by color formation (e.g., MBTHSB-ANS), surfactants may be added to brighten the color and enhance the contrast with the uncolored surround.

Organic solvents may also be employed in the practice of this invention and may be included in the formulation of the testing reagent for the matrix, provided, of course, that they are compatible with the matrix and testing reagent

compositions. Potentially suitable organic solvents include chloroform, acetone, alcohols, methylene chloride, diethyl and petroleum ethers, acetonitriles, and mixtures thereof. In the practice of the present invention, 70% ethanol in water is particularly preferred.

The testing reagent that is coated on or impregnated into the matrix is not uniform over the surface of the test strip. Instead, the reagent is preferably applied to the matrix in a series of parallel stripes, or "segments," that extend across the narrow dimension of the strip. The composition in adjoining segments increases, stepwise, in inhibitor concentration. Each segment has a bibulous assay area. It is in the assay areas that the testing reagent reacts with any glucose in the blood to cause a color change, provided that the glucose concentration is large enough to overcome the inhibitor level in that assay area. Thus, each succeeding assay area requires, stepwise, a greater glucose concentration in the sample to cause the area to change color.

Optionally, one of the assay areas is adapted to serve as a timer, to indicate that enough time has elapsed for the reagent to react with the glucose on each of the assay areas. The timer segment of the matrix is coated or impregnated with a composition that consists of the testing reagent and, in addition, glucose. Since the testing reagent's purpose is to change color in response to glucose, combining the two without causing the color change requires some care. An amount of inhibitor beyond that required for the timing function must be present to compensate for this effect. The rate at which the timer segment is dried, after the glucose containing solution is applied, is controlled. In practice, the membrane is first coated with a solution containing buffers, stabilizers, and enzymes, and that coating is dried to form a first layer. Then, a second coating pass applies a solution containing indicator, inhibitor, and glucose. Parameters such as web speed, oven temperature and airflow, and quantity of coating solutions deposited will have been fixed beforehand and appropriate adjustments made to the inhibitor and/or glucose concentrations. Instead of applying the second coating directly, an alternative, less preferred, involves making the second coating on a separate web and then placing it over the first layer.

When a sample is applied to the strip, hydration of the timer segment composition permits the color-forming reaction to proceed. The time it takes for the timer segment to change color is then determined by the temperature and by characteristics of the testing reagent, particularly the inhibitor concentration, the amount of glucose, and the hydration and oxygen diffusion rates.

The timer color-change time can be made to depend on the glucose concentration in the sample or, alternatively, to be independent of that concentration. By incorporating a great excess of glucose in the timer, the time is substantially independent of the sample's glucose concentration. By incorporating less glucose in the timer, the time may be made to depend on the glucose in the sample; i.e., the timer will change color faster if glucose concentration in the sample is greater. Preferably, the glucose concentration in the timer is greater than about 1500 mg/dL, which makes the timer substantially independent of the sample glucose concentration in the range from about 40-400 mg/dL. The timer segment composition includes excess amounts of the component (such as glucose oxidase) that converts glucose to hydrogen peroxide and of glucose. The timer composition should then include at least as much, or more, inhibitor than does the result segment that has the highest inhibitor concentration (which corresponds to the highest glucose reading).

The timer also serves an important quality-control function, by making it apparent when a test strip has been compromised by exposure to moisture. The test strip must remain dry until the time it is to be used, because components that convert glucose to hydrogen peroxide (generally enzymes) tend to degrade on exposure to moisture. Thus, if the strip is prematurely exposed to moisture, it will become compromised. But the impairment of the test strip is not apparent to a user, who may, therefore, use such a strip and get an erroneous result. However, if the strip includes a timer segment, exposure to moisture causes the timer to change color, which alerts the user to the fact that the strip has been compromised and should not be used.

In addition to the reagent-containing matrix, the strip of the present invention includes a bottom layer that supports the matrix. The bottom layer is preferably a thermoplastic sheet, more preferably a polyester, and has a hole through which sample may be applied to the sample side of the matrix. From the sample hole the blood sample is distributed along the length of the matrix. If the bottom layer is generally opaque, then one or more transparent window sections may be located an appropriate distance from the sample hole, the appearance of sample in the window(s) confirming that adequate sample has been applied to the strip.

Distributing the blood from the sample hole to the assay areas involves an intermediate layer that lies between the bottom layer and the membrane and, optionally, is adhered to both of them. The intermediate layer is preferably a thermoplastic sheet, more preferably a polyester. It has cutouts that guide the sample down the length of the strip along non-bibulous paths on the membrane and that direct the sample to each of the assay areas. Notches in the intermediate layer align with the assay areas, so that each assay area is substantially surrounded by the walls of the intermediate layer.

A preferred structure for the non-bibulous paths on the membrane is formed by collapsing the membrane pore structure. That can be accomplished by heating, either directly or by using a laser or ultrasound, and preferably including pressure. However, the preferred method is crushing. Thus, the membrane is crushed to make it non-bibulous (but still hydrophilic) everywhere, except for the assay areas. For the preferred membranes of this invention, crushing is pref-

erably accomplished by applying high pressure - at least 6 tons/in² (80,000 kPa) - and, optionally, heat (at least 110°C) to the membrane areas to be crushed. The preferred pressures and temperatures depend, of course, on the crush mechanism and dwell time, as well as the membrane parameters. Optimum values can be determined by routine experimentation.

For precise measurements, it is important that the volume of blood provided to each assay area be reproducible. If the notches entirely encircled the assay areas, then, assuming a liquid-tight seal between the intermediate layer and both the bottom layer and the crushed membrane, each assay area would be associated with a closed (cylindrical) volume whose walls are formed by the intermediate layer and whose ends are formed by the membrane and bottom layers. However, a distribution channel runs along the strip and feeds sample to each of the assay areas. High precision requires that the distribution channel provide a fixed volume of sample to each assay area but then provide no more, at least not in the time frame of the measurement - about 1 or 2 minutes. Since the initial sample volume is variable, there is preferably an absorbent layer at each end of the membrane to carry off excess sample from the ends of the distribution channel. Absorbent layers at the ends of the channel also enhance wicking of the sample along the length of the strip. Nonwoven fabrics, well known in the art, form the preferred absorbent layers.

The color change caused by glucose in the test sample appears on the testing side of the membrane. It is convenient to overlay that side of the membrane with an upper layer that has holes which align with the assay areas. The holes make the color changes visible and also permit oxygen to reach the reaction sites. The upper layer may be attached to the membrane, for example, with an adhesive. Any adhesive is preferably limited to non-bibulous areas of the membrane, if it would interfere with the glucose-measuring reactions. However, if the adhesive doesn't interfere with the reactions, its placement is less critical.

Since the assay areas, when they contain the preferred reagent, slowly undergo a color change when exposed to light or oxygen and since the optional timer is sensitive to moisture, strips are preferably packaged in an opaque oxygen- and moisture-impermeable enclosure, such as a sealed foil wrap. If strips are individually packaged, the strip may remain in the peeled-open wrap during use.

The invention will now be described further with reference to the Figures. FIG. 1 shows a matrix 10 of the present invention, for measuring the amount of analyte in a biological fluid. Although shown in an arched position, matrix 10 is flexible and is generally in a flat plane when used. The matrix includes a sample side 12, to which the biological fluid sample is applied, and a testing side 14, on or near which a change in color indicates the presence of the analyte. The color change results from the interaction of the analyte with reagent impregnated in pores 16. Preferably, for measuring the concentration of glucose in blood, pore sizes are relatively large near sample side 12 and decrease in size as testing side 14 is approached. The pore size gradient serves to trap red blood cells near sample side 12, so that their color does not interfere with the ability to see the color change that indicates the presence of the analyte.

Three parallel segments, a, b, and c, are shown schematically. Each succeeding segment has stepwise more inhibitor than the one before. In a preferred embodiment, after reagent has been applied to the membrane in parallel segments, as shown, the membrane is crushed everywhere but in the assay areas, where the analyte-reagent reactions take place. This pattern of bibulous assay areas - a single area located in each of the parallel segments - and non-bibulous crushed areas is depicted in the plan view of Fig. 2 and the enlarged fragmentary perspective view of Fig. 3.

Fig. 2 is a bottom plan view, in partial cutaway, of the sample side 12 of membrane 10 and absorbent layers 20 and 22, overlaid with intermediate layer 24 and bottom layer 26. Membrane 10 and absorbent layers 20 and 22 are preferably supported by a top layer, not shown. Absorbent layers 20 and 22 are preferably located at the ends of the membrane (beyond dashed lines A and B) to absorb blood sample that is in excess of the volume needed for the measurement. That volume must be sufficient to provide sample to each of the assay areas and, if present, the timer area as well. In general, a strip that has fewer assay areas doesn't require as much sample, but provides a smaller range of glucose values and/or less precision. Fig. 2 shows 9 bibulous areas, representing 8 assay areas (numbered 1-8) and a timer (T), which provides adequate range and precision while not requiring unacceptably large sample volume. Intermediate layer 24 has a notch 28, which aligns with sample hole 30 in bottom layer 26. Sample is introduced through sample hole 30 and is directed by capillary action along central channel 32 of intermediate layer 24 to each of the assay areas and the timing area, any excess sample being absorbed in absorbent layers 20 and 22. The appearance of sample through optional clear windows 34 and 35 confirms that sufficient sample has been provided for measurement. Preferably, intermediate layer 24 forms a seal with sample side 12 of the membrane, so that sample cannot, for example, flow directly between adjoining assay areas.

FIG. 3 is an enlarged fragmentary perspective view, depicting parts of 3 assay areas, 6, 7, and 8, seen through bottom layer 26, and separated by fingers of intermediate layer 24. Optional adhesive layers 24A join intermediate layer 24 to bottom layer 26 and membrane 10. Vent holes 40 in layer 26 facilitate sample flow into the strip. Holes, such as 38, in top layer 36 line up with the bibulous areas, making visible any color change in the bibulous area and also admitting oxygen needed for the color-changing reaction. Optional adhesive layer 36A joins top layer 36 to the testing side of membrane 10.

Fig. 4 is a cross section taken along line 4-4 of Fig. 2, which shows top layer 36, in addition to the layers shown

in Fig. 2. Vent holes in bottom layer 26, such as 40, line up with the assay and timer areas and facilitate sample filling the volume surrounding each of those areas. The volumes to be filled are bounded by membrane 10, intermediate layer 24 and bottom layer 26. Note that the separation between the top of assay area 3 and bottom layer 26 is only about 12 micrometers but is shown larger than to scale for clarity.

Fig. 5 is a bottom plan view of a strip of the present invention, showing sample hole 30 and the graphics that direct the user to introduce the sample through that hole. When sample is seen through clear windows 34 and 35, it confirms that adequate sample has been applied to the strip.

Fig. 6 is a plan view of top layer 36 of a strip that has been calibrated to associate assay areas with glucose concentration.

Fig. 7 shows the strip of Fig. 6 after a blood sample has been applied to opening 30 (Fig. 2), the sample has spread along central channel 32, and glucose in the sample has reacted with the reagent in the assay areas. Since the bottom assay area has the least inhibitor, that area will have changed color first. Thereafter, the second and then the third area changed color. The upper circles did not change color, because there was too little glucose in the sample. Since enough time has elapsed for timer area 42 to change color, the strip can be read. Thus, the result depicted in FIG. 7 indicates that the sample glucose concentration is at least 120 mg/dL, but less than 150 mg/dL. The reading can be taken at any time after timer area 42 changes color. Note that in FIG. 7 the color change caused by the reaction with glucose is from white to colored. However, the system could alternatively operate with an indicator dye that is destroyed by the glucose-induced oxidation, with a corresponding color change from colored to white.

For a better understanding of the present invention, the following Examples further illustrate various embodiments of the invention. The Examples are not intended to be in any way limiting.

EXAMPLE 1 - BPR INDICATOR

The following solution was prepared:

		Enzyme Solution	
Distilled Water	83.5 g	0.2M Aconitic Acid	27.0 g
1% (w/w) EDTA Na ₂	23.8 g	Glucose Oxidase	165,000 U
Aconitic Acid	6.0 g	HRPO	340,000 U
NaOH (solid)	2.2 g		
Crotein SPA	4.2 g		
Imidazole	0.6 g		
Mannitol	3.0 g		
5% (w/w) Surfactol Q1	3.0 g		
Adjust pH to 4.80 Ethyl Alcohol	40.0 g		
PPG-410	5.6 g		
Enzyme Solution	28.0 g		

Memtec BTSH 55 membrane was immersion coated in this solution and the excess doctored off with glass rods. The coated membrane was dried in a flotation dryer at 180F under moderate airflows so that the web was substantially dry within 20 seconds. The web was spooled in preparation for the second coating, described below.

The following solutions were prepared:

Ascorbate (inhibitor) stock solution		Diluent	
Distilled Water	190 g		370 g
1% EDTA Na ₂	55 g		107 g
BPR	0.36 g		0.71 g
PolyQuart® H	6 g		11.8 g
PPG-410	14.2 g		27.8 g
Ascorbic Acid	1.37 g		--
Ethyl Alcohol	243 g		477 g

EP 0 759 555 A2

(continued)

Ascorbate (inhibitor) stock solution			Diluent
Timer Solution			
Diluent (per above formula)		120 g	
Ascorbic Acid		0.885 g	
Glucose Solution*		17.25 g	

* The Glucose Solution is a 16.0 g/dL solution of glucose in water allowed to mutarotate for 24 hours, stored refrigerated.

The following dilutions of the stock solution were made:
 0.0405:1, 0.108:1, 0.236:1, 0.369:1, 0.569:1, 1.260:1. This stepwise increase in inhibitor concentration corresponds to the stepwise-greater glucose concentration that the assay areas report. These solutions, along with the timer solution, were coated side-by-side onto the large-pore side of the enzyme-loaded membrane so as to deposit approximately 1.2×10^{-1} mL per square millimeter of membrane. The membrane was wet approximately fifteen seconds before experiencing the same drying conditions as described above for the enzyme coating step. Results showed the timer reacting in about 70 seconds with about 95% of results falling between 64 and 79 seconds.

EXAMPLE 2 - MBTHSB-ANS INDICATOR

The following solution was prepared:

HPLC water	1500 mL
Citric Acid	16.92 g
Sodium Citrate	20.88 g
Mannitol	15 g
Disodium EDTA	1.26 g
Gantrez S95	6.75 g
Croton SPA	36 g
Glucose Oxidase	1.69 MU
HRPO	1.5 MU
Carbopol 910*	75 mL
Disodium Citrate**	225 mL

* 11% solution in Acetonitrile

** 0.1M, pH 5.0

Memtec BTS 35 membrane was coated in a trough so that the large-pored surface contacted the coating solution; excess solution was removed with glass rods as before. The membrane was dried and spooled as in Example 1. The following solutions were made:

Solution A (Indicator)		Solution B (Wetting Agent)	
70% (v/v) Ethanol	2819 mL	Maphos® 60A	41 g
MBTHSB	2.98 g	70% (v/v) Ethanol	205 mL
(NH ₄) ANS	25.83 g		
Solution B	205 mL		
2% DTPA	51.25 mL		
Solution C (Ascorbate Stock)		Solution D (Timer)	
Water	115 mL	Water 53 mL	
Ascorbic Acid	4.58 g	Ascorbic Acid	8.75 g
Ethanol	267 mL	Ethanol	123 mL
		Bring volume to 175 mL with 70% EtOH	
		Glucose Solution	40.5 mL

For each inhibitor solution, the volume of Solution A was fixed at 263 mL. For the various assay areas, the ratio of 70% EtOH:Solution C was varied from 58.9 to 0.200 so that the volume of 70% EtOH + Solution C added to Solution A was 87.5 mL for all inhibitor solutions. This effectively altered only the concentration of inhibitor in each solution. The solutions containing the stepwise-increasing inhibitor concentration and the timer solution (Solution D) were coated side-by-side onto the large-pore side of the membrane. Deposition rate was adjusted to achieve $\sim 8 \times 10^{-5}$ mL of inhibitor per square millimeter of membrane. The membrane was dried as above, except that the delay between coating and drying was about 1.6 minutes. Results showed the timer reacting in about 60 seconds with little effect from blood hematocrit from 30 to 55% or glucose from 78 to 420 mg/dL.

It will be understood by those skilled in the art that the foregoing description and Examples are illustrative of practicing the present invention but are in no way limiting. Variations of the detail presented herein may be made without departing from the scope and spirit of the present invention.

Claims

1. An elongated multilayer reagent test strip for measuring the concentration of an analyte, such as glucose, in a sample of biological fluid, such as blood, that is applied to the strip, comprising:

a) a bottom layer with a through hole for accepting the sample;

b) a membrane layer, having a sample side facing the bottom layer and a testing side opposite to it, and containing a reagent that can react with the analyte to produce a color change, the reagent comprising

i) a first component (such as glucose oxidase) that interacts with the analyte to form hydrogen peroxide;

ii) a second component (such as a peroxidase and an indicator dye or dye couple that changes color when it is oxidized) that interacts with the hydrogen peroxide to undergo a color change; and

iii) a third component (such as ascorbic acid) that inhibits the change in color of the second component;

c) an intermediate layer between the bottom and membrane layers; and

d) metering means for distributing sample along the strip, the metering means comprising

i) a non-bibulous region in the membrane layer; and

ii) a fluid transport channel formed in the intermediate layer for guiding sample over the surface of the non-bibulous region to a plurality of discrete bibulous assay areas arrayed along the length of the membrane layer;

the inhibitor concentration increasing in a predetermined way with distance from a first end of the strip, so that a correspondingly increasing analyte concentration must be contained in a sample if it is to effect a color change, whereby one or more assay areas may change color when a sample is applied to the strip, and the color-changing area most distant from the first end indicates the analyte concentration in the sample.

2. The strip of claim 1 in which the bottom layer further comprises a plurality of through holes in alignment with the assay areas.

3. The strip of claim 1 or claim 2 in which the membrane layer comprises an anisotropic porous membrane having pores that are larger near the sample side and smaller near the testing side.

4. The strip of claim 3 in which the pore sizes are selected so that the red blood cells of a whole blood sample are trapped in the membrane.

5. The strip of any one of claims 1 to 4 in which the intermediate layer and/or the bottom layer comprises a thermoplastic sheet.

6. The strip of any one of claims 1 to 5 in which the bibulous and non-bibulous regions comprise uncrushed and crushed regions of the membrane layer, respectively.

7. The strip of any one of claims 1 to 6 further comprising a top layer that is contiguous with the top surface of the membrane layer and has through holes that align with the assay areas.

8. The strip of any one of claims 1 to 7 further comprising absorbent layers that contact each end of the membrane.

9. The strip of any one of claims 1 to 8 further comprising a timer element, which comprises an assay area that includes, in addition to the reagent, an amount of glucose that causes the area to change color a predetermined time after the sample is applied to the strip.

10. A method for measuring the concentration of an analyte in a sample of biological fluid, comprising the steps of:

(a) applying the sample to a reagent test strip that comprises:

(i) a plurality of bibulous assay areas that each change color when contacted with fluid containing at least a predetermined amount of analyte, greater than the amount of analyte that causes a change in color of the assay areas that are closer to a first end of the strip and

(ii) metering means for distributing the sample along a predetermined non-bibulous path to each of the assay areas and

(b) observing the assay area that changes color and that is most distant from the first end of the strip.

FIG. 1

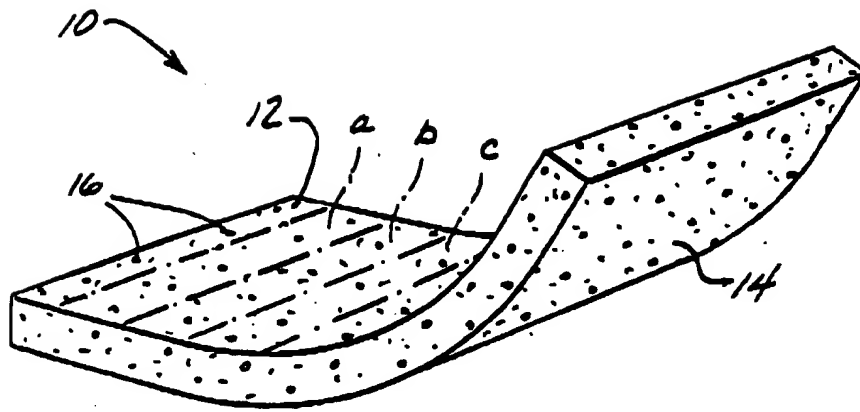
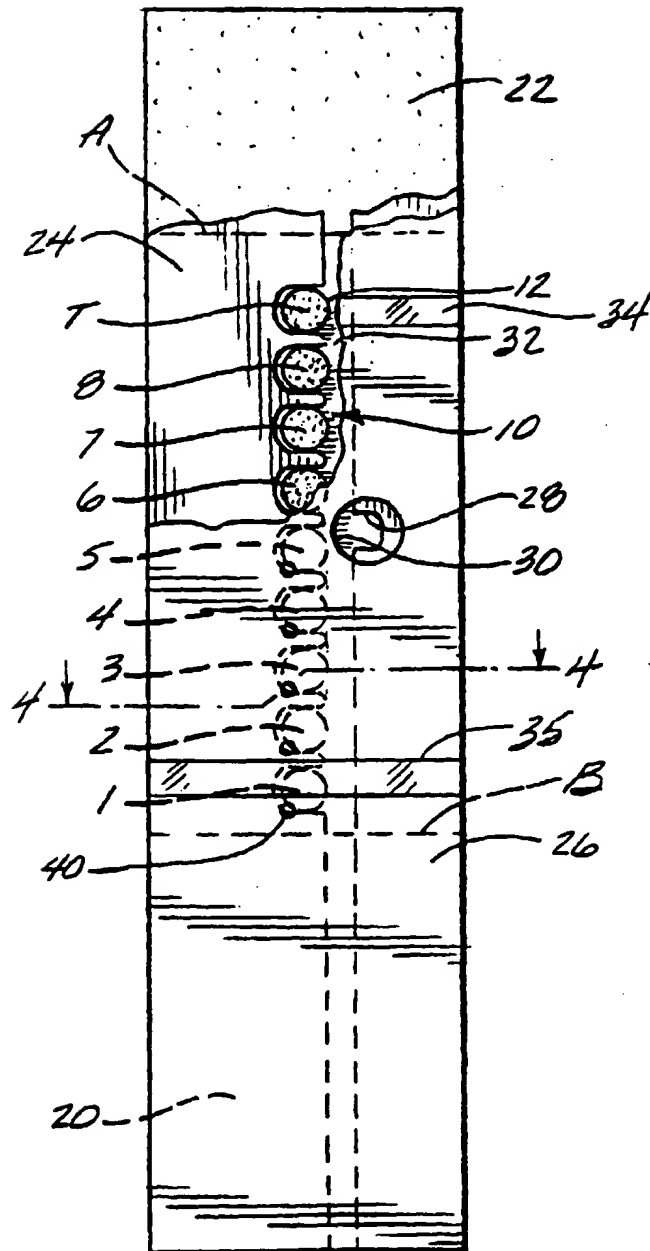


FIG. 2



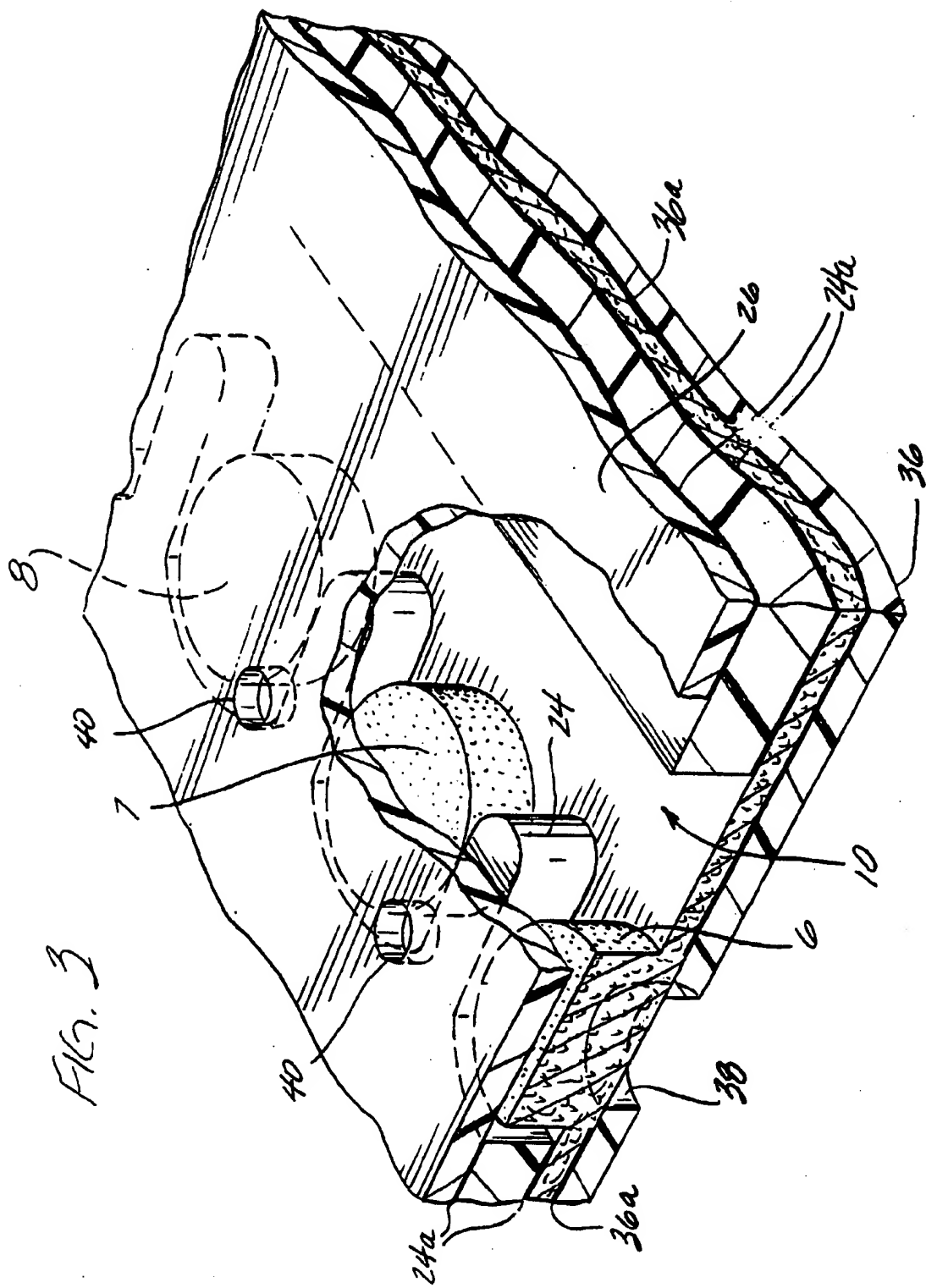


FIG. 4

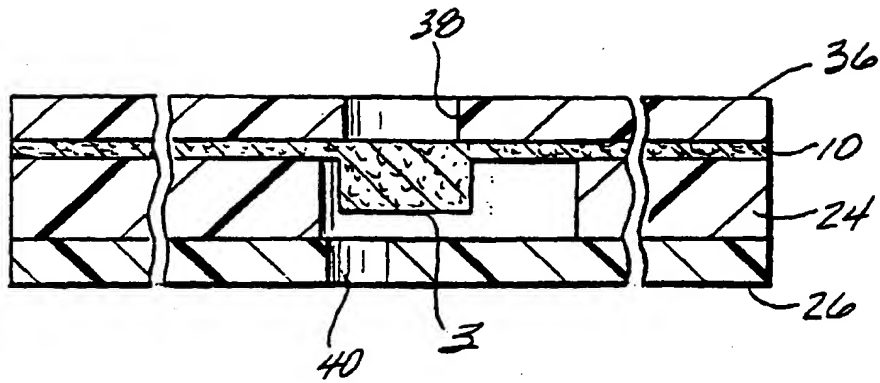


FIG. 5

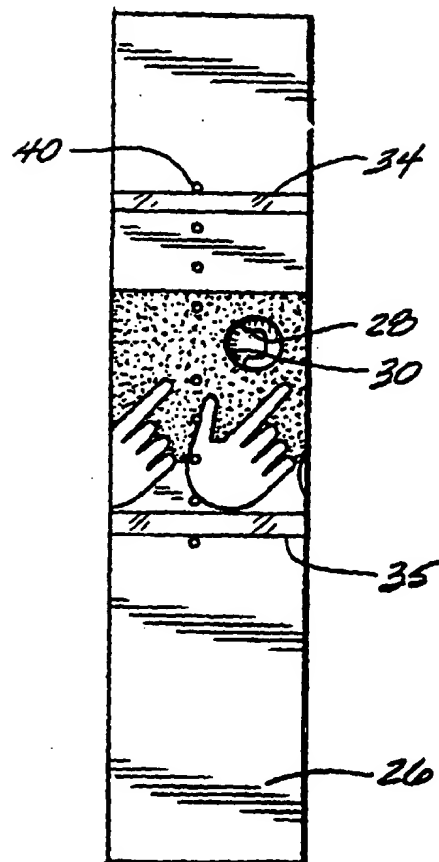


FIG. 6

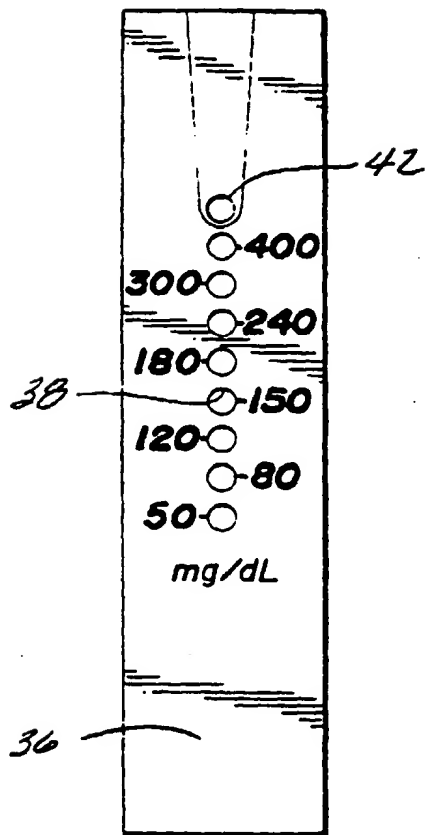
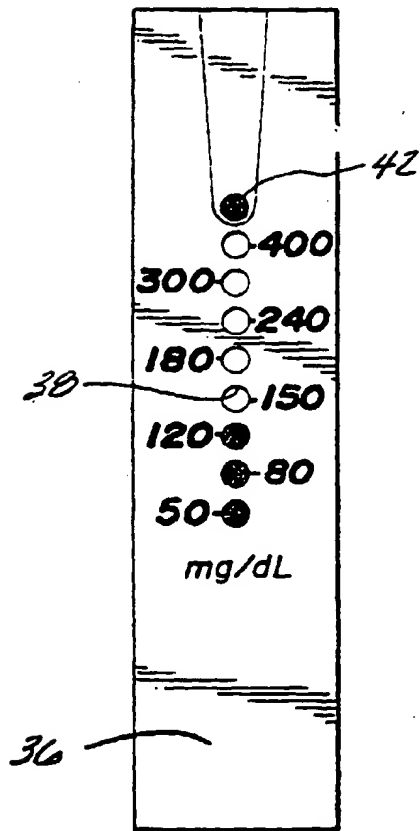


FIG. 7





(12) **EUROPEAN PATENT APPLICATION**

(88) Date of publication A3:
08.07.1998 Bulletin 1998/28

(51) Int Cl.⁶: **G01N 33/52**

(43) Date of publication A2:
26.02.1997 Bulletin 1997/09

(21) Application number: **96305720.3**

(22) Date of filing: **02.08.1996**

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU NL PT SE

(30) Priority: **03.08.1995 US 528511**

(71) Applicant: **LIFESCAN, INC.**
Milpitas California 95035 (US)

(72) Inventors:
• **Douglas, Joel**
Santa Clara, CA 95051 (US)
• **Tomasco, Michael F.**
Mountain View, CA 94041 (US)

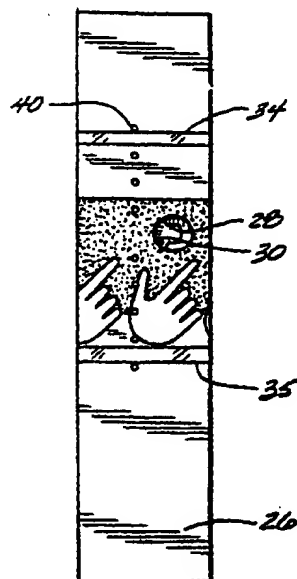
- **Kiser, Ernest**
Los Altos, CA 94024 (US)
- **Dato, Remedios**
Pleasanton, CA 94588 (US)
- **Rice, Edward G.**
Palo Alto, CA 94303 (US)
- **Tuohy, Deborah P.**
San Jose, CA 95120 (US)
- **Maxson, Mark**
San Jose, CA 95148 (US)

(74) Representative: **Mercer, Christopher Paul et al**
Carpmaels & Ransford
43, Bloomsbury Square
London WC1A 2RA (GB)

(54) **Direct-reading reagent test strip**

(57) A multilayer reagent test strip measures the concentration of analyte in a liquid sample that is applied to it. The sample is guided to a number of assay areas arrayed along the strip, where the analyte can react with a reagent to cause a color change. Each assay area also includes an inhibitor for the color-change reaction. The inhibitor concentration increases in successive assay areas; thus, the number of areas that change color is a measure of the analyte concentration. The test strip is particularly adapted for measuring glucose in a whole blood sample. In a preferred embodiment, the sample is guided to the assay areas along a path formed by crushing selected areas of a membrane, and the assay areas are uncrushed areas of the membrane.

FIG. 5





European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 96 30 5720

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	EP 0 475 692 A (LIFESCAN INC) 18 March 1992 * abstract; figures 6,7A-C * * page 6, line 38 - line 40 * * page 7, line 42 - line 57 * * page 9, line 15 - line 20 * * page 9, line 42 - line 47 *	1,3,4,10	G01N33/52
D	& US 5 306 623 A ---		
D,X	US 3 964 871 A (HOCHSTRASSER HARRY) 22 June 1976 * abstract; examples * ---	1,2,5,10	
A	WO 91 11530 A (CRANFIELD BIOTECH LTD) 8 August 1991 * page 2, column 3 - page 5, column 3 * * page 8, column 4 - page 9, column 2; example 1 * ---	1,8,10	
E	EP 0 735 369 A (LIFESCAN INC) 2 October 1996 * the whole document * ---	1,2,5-10	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
E	EP 0 769 558 A (LIFESCAN INC) 23 April 1997 * the whole document * -----	1-8,10	G01N
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 19 May 1998	Examiner Ceder, O
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03 82 (P04C01)